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mtDNA Depletion with Variable Tissue Expression: A Novel Genetic Abnormality in Mitochondrial Diseases

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Summary

We studied two related infants with a fatal mitochondrial disease, affecting muscle in one and liver in the other. Quantitative analysis revealed a severe depletion of mtDNA in affected tissues. This genetic abnormality was also observed in muscle of an unrelated infant with myopathy and in muscle and kidney of a fourth child with myopathy and nephropathy. Biochemistry, immunohistochemistry, and in situ hybridization showed that the depletion of mtDNA in muscle fibers was correlated with a respiratory chain defect and with lack of mitochondrially translated proteins. Although the differential tissue involvement in these infants suggests mtDNA heteroplasmy, sequence analysis of mtDNA replication origins did not reveal any abnormality that could account for the low copy number.

Introduction

Several mutations of human mtDNA have been identified in patients with mitochondrial encephalomyopathies, a clinically heterogeneous group of diseases defined by morphologically abnormal mitochondria, defects in mitochondrial metabolism, or maternal mode of inheritance (Holt et al. 1988; Wallace et al. 1988; Poulton et al. 1989; Holt et al. 1990; Shoffner et al. 1990). Heteroplasmic deletions of mtDNA have been invariably associated with paralysis of extraocular muscles (progressive external ophthalmoplegia, or PEO) and abnormal proliferation of mitochondria manifested as ragged red fibers (RRF) in muscle sections stained with the modified Gomori trichrome (Holt et al. 1989; Moraes et al. 1989; Nelson et al. 1989). Most patients with single deletions of mtDNA have been sporadic (Holt et al. 1989; Moraes et al. 1989).

We studied an infant girl (patient 1), originally described by Boustany et al. (1983), who developed a fatal mitochondrial myopathy characterized by progressive generalized hypotonia, PEO, and severe lactic acidosis. About 1 year later, a 2½-mo-old sister of the patient died with similar clinical, morphological and biochemical abnormalities, but, unlike her sister, she also had cytochrome *b* deficiency in liver (Aprille 1985). A second cousin of these two patients, related through the maternal grandfather (fig. 1, patient 2), died at age 9 mo of hepatic failure with mitochondrial abnormalities similar to those observed in muscle of her cousins.

The morphological and biochemical changes in mitochondria from the two siblings and the cousin suggested that the same genetic defect was responsible for the clinical manifestations, but the different organ involvement (only muscle in the first patient, liver and muscle in her sister, and liver but probably not muscle in the cousin) seemed to defy rational genetic explanation. Because differential tissue involvement in mitochondrial diseases has been associated with random segregation of wild-type and mutant mtDNAs (heteroplasmy) (Moraes et al. 1989; Nelson et al. 1989; Rotig et al. 1989; Shanske et al. 1990; Zeviani et al. 1990), we looked for mutant mitochondrial genomes in the tissues of patients 1 and 2 (tissues from the affected sibling of patient 1 were no longer available). We found no qualitative alteration but discovered a severe quantitative defect of mtDNA in affected tissues.

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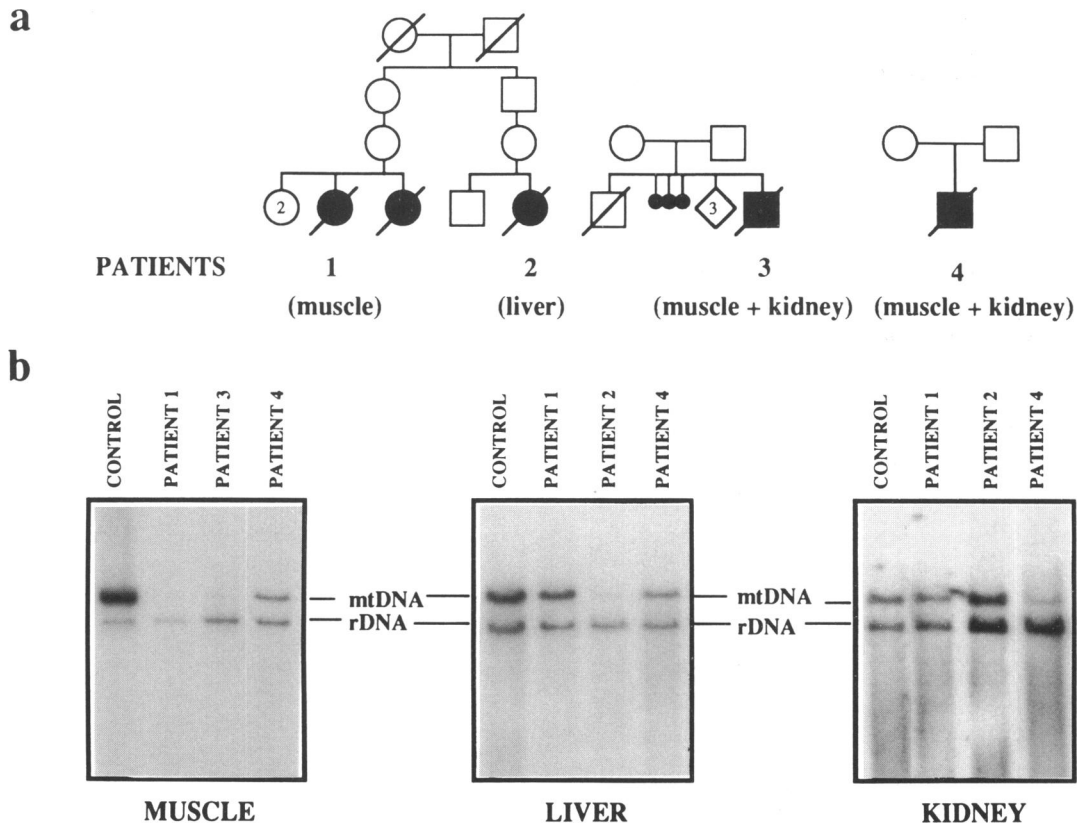

Figure 1 Depletion of mtDNA in clinically affected tissues of infants with mitochondrial diseases. *a,* Pedigrees of four patients. Affected tissues are indicated in parentheses. *b,* Autoradiograms of Southern blots of total DNA extracted from indicated tissues. Total DNA was digested with *Pvu*II, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose (Zeviani et al. 1988), and hybridized simultaneously with two ^{32}P-labeled probes: total human mtDNA and a cloned fragment of the human 18S ribosomal RNA gene.

Screening muscle biopsies from other infants with similar clinical presentations, we identified two more patients with mtDNA depletion.

Patients and Methods

Patients

Patients 1 and 2 have been described elsewhere (Boustany et al. 1983; Aprille 1985; in the latter reference, patient 1 is also patient 1 in this report, patient 2 is the affected sister mentioned but not studied here, and patient 3 is the cousin designated patient 2 in this report). Briefly, patient 1 developed, soon after birth, progressive generalized hypotonia, PEO, and severe lactic acidosis. She died at 12 wk of respiratory failure. Electron microscopy of a muscle biopsy and of postmortem muscle showed increased numbers of mitochondria with unusual morphology. Biochemical studies of isolated muscle mitochondria showed cytochrome *c* oxidase (COX) deficiency and absence of cytochromes *b* and aa_3. Liver, kidney, brain, heart, and lungs were clinically unaffected (except for hepatomegaly late in the course) and had normal COX activity and cytochrome contents. About 1 year later, a 2½-mo-old sister of the patient died with similar clinical manifestations. Biochemical analysis revealed lack of COX activity and absence of cytochromes *b* and aa_3 in muscle, but she also had cytochrome *b* deficiency in liver (Aprille 1985). A second cousin of these two patients, related through the maternal grandfather (fig. 1, patient 2), died at age 9 mo of hepatic failure. Postmortem examination showed abnormal mitochondrial morphology and proliferation in liver with deficient COX activity and absence of cytochromes *b* and aa_3. Kidney, the only other tissue available for study, was clinically and pathologically normal. Other organs, including muscle, were clinically spared as well

(Boustany et al. 1983). There was no history of consanguinity in the family of these three children.

Patient 3 was the last of four children of a nonconsanguineous Turkish couple. The second child had died 6 h after birth of "heart failure," and the mother had had three miscarriages. Our patient was diffusely weak and hypotonic from birth, and at 8 wk of age was found to have lactic acidosis (venous blood lactate 8 mM; normal <2 mM). He also had renal dysfunction, with De Toni-Fanconi-Debré syndrome. Weakness of respiratory muscles made assisted ventilation necessary. He developed seizures with encephalographic evidence of hypsarrhythmia. A computed tomography scan of the brain was normal. In the last months of life he developed congestive heart failure, and died at 7 mo of age of cardiac arrest. Autopsy was not performed. A muscle biopsy performed at 5 mo of age showed numerous RRFs.

Patient 4 was the first child of healthy nonconsanguineous parents. He was born at term after a normal pregnancy. At 1 wk of age, he developed rapid breathing and was found to have metabolic acidosis and De Toni-Fanconi-Debré syndrome. He was weak, hypotonic, lethargic, and areflexic. There was no response to the doll's-eyes maneuver. He required assisted ventilation on three occasions and became ventilation-dependent at 2 mo of age. Blood lactic acid was 5 mM (normal <2 mM); total and free carnitine were markedly decreased (13 and 3 nmoles/ml; normal 47 ± 10 and 37 ± 8) but esterified carnitine was normal. A muscle biopsy showed abundant RRFs and lipid storage, and COX deficiency was documented. Therapy with carnitine, ascorbic acid, menadione, and methylene blue had no effect. Heart and lung were normal, and the liver was slightly enlarged. Blood lactic acid increased gradually from 5 to 10.5 to 16.6 mM. He remained floppy and ventilator dependent and died of an intercurrent infection at 9 mo of age. At autopsy, skeletal muscle had the same mitochondrial changes shown in the biopsy but, in addition, a moderate degree of fibrosis. Heart, liver, and brain were normal.

Southern Blot Analysis

In order to quantify mtDNA levels, approximately 5 μg of total DNA extracted from different tissues was digested with *Pvu*II, electrophoresed through a 0.8% agarose gel, and transferred to nitrocellulose as described elsewhere (Zeviani et al. 1988). The filters were hybridized simultaneously with two probes. One probe was the entire human mitochondrial genome (16.6 kb) isolated as described elsewhere (Zeviani et al. 1988). The other probe was a 5.8-kb *Eco*RI insert from a cloned fragment (pB) containing nuclear-encoded 18S rDNA sequences (Wilson et al. 1978). Probes were prepared by the random primer labeling kit (Boehringer Mannheim). Filters were hybridized with 2×10^5 cpm/ml of mtDNA probe (specific activity of approximately 6×10^8 cpm/μg) and 4×10^5 cpm/ml of rDNA probe (specific activity of approximately 6×10^7 cpm/μg). Final washings were done in 1 × SSC and 0.1% SDS. Hybridization and washings were performed at 65°C. The nitrocellulose filters were scanned with a Betascope 603 blot analyzer (Betagen). mtDNA (16.6-kb) and rDNA (12.0-kb) signals were determined after subtracting background counts taken from regions flanking the hybridizing bands in each lane. For each tissue, we compared samples from patients and controls on the same membrane. Equal amounts of ^{32}P-labeled probes (with the same specific activity) were used in all experiments. α-[^{32}P]-dATP was from New England Nuclear. Restriction enzymes were from Boehringer Mannheim, and chemicals were from Sigma.

Histology and Immunohistochemistry

COX cytochemistry, and immunostaining of frozen muscle sections with antibodies against subunits II and IV of COX, were performed as described elsewhere (Mita et al. 1989).

A monoclonal anti-DNA antibody (Chemicon MAB031) was used to visualize mtDNA in frozen muscle sections. The ability of this antibody to recognize mtDNA was assessed by double labeling using different fluoresceins in immunostaining of frozen muscle sections with anti-DNA and anti-COX II. The same cytoplasmatic network staining corresponding to mitochondria was observed with both antibodies (F. Andreetta and E. Bonilla, unpublished data).

In situ Hybridization

In situ hybridization for DNA detection using ^{32}P-labeled probes, at the specific activity described above, was performed essentially as described by Mita et al. (1989).

Biochemistry

For patients 1 and 2, mitochondrial enzymes were measured in whole homogenates as described by Boustany et al. (1983). For patients 3 and 4, mitochondrial enzymes were measured in homogenate supernatants after centrifugation at 750 *g* for 15 min (DiMauro et al. 1987).

DNA Amplification and Direct Sequencing

The origins of mtDNA replication were amplified by PCR (Saiki et al. 1988) using the Ampli-Taq kit (Perkin-Elmer). Amplifications and direct sequencing of PCR products were performed as described elsewhere (Mita et al. 1990). Primers used in the amplification and sequencing of the origin of replication of the H-strand were (positions according to Anderson et al. 1981) Alu 580B (mtDNA positions 571–592), Dra 16030F (16000–16023), 140F (130–150), and Ssp 175F (163–184). Primers used in the amplification and sequencing of the origin of replication of the L-strand were Eco 5260F (5260–5288) and Stu 5870B (5827–5849).

Results

Southern blot analysis of muscle mtDNA in patient 1 showed no gross abnormalities (deletions or duplications), but the 16.6-kb band corresponding to normal mtDNA was barely discernible under conditions in which mtDNA was readily detected in normal muscle. To quantify the levels of mtDNA, genomic blots of restriction digests of total DNA were hybridized simultaneously with mtDNA and with nuclear 18S ribosomal DNA gene probes. The rDNA genes are normally present in hundreds of copies in each cell (Bross and Krone 1972; Schmickel 1973; Young et al. 1976; Schmickel and Knoller 1977), providing a suitable control for quantitation of mtDNA. Using the 18S rDNA signal as internal control, we found that mtDNA levels in muscle of patient 1 were 2% of control values (fig. 1, table 1). The same pattern was observed with other restriction enzymes (*Hin*dIII and *Bam*HI; not shown), ruling out the possibility of a *Pvu*II polymorphism. Similar studies in multiple postmortem tissues from patient 1 showed that the severe depletion of mtDNA was restricted mainly to skeletal muscle: mtDNA levels were normal (within one SD of control values) in kidney, liver, brain, and fibroblasts (fig. 1, table 1). These findings prompted us to analyze tissues from patient 2, the cousin who had died of hepatic failure. Liver mtDNA from patient 2 was approximately 12% of normal values, and mtDNA levels were normal in kidney (fig. 1, table 1). No other tissue was available from patient 2. Biochemical anal-

Table 1

Correlation of Genetic, Biochemical, and Morphological Features in Infants with mtDNA Depletion

Patient and Tissue	mtDNA/rDNA			COX Activity			Mitochondrial Proliferation[c]
	Patient	Control (n)[a]	% of Control	Patient	Control (n)[b]	% of Control	
1:							
Muscle.....	.11	5.47 ± 2.63 (8)	2	4	184 ± 121 (13)	2	+
Liver	1.54	1.52 ± .46 (8)	101	98	174 ± 72 (16)	56	–
Kidney.....	.50	1.00 ± .58 (6)	50	87	140 ± 55 (4)	62	ND
Brain.....	1.06	1.60 ± .56 (9)	66	98	60 ± .41 (4)	163	ND
Fibroblasts...	1.02	.79 ± .13 (3)	128	ND	ND	ND	ND
2:							
Liver	.18	1.52 ± .46 (8)	12	12	174 ± 72 (16)	7	+
Kidney.....	.63	1.00 ± .58 (6)	63	63	140 ± 55 (4)	44	ND
3:							
Muscle.....	.18	5.47 ± 2.63 (8)	3	.02	2.80 ± .52 (71)	1	+
4:							
Muscle.....	.94	5.47 ± 2.63 (8)	17	.14	2.80 ± .52 (71)	5	+
Liver	1.01	1.52 ± .46 (8)	66	1.68	1.62 ± .51 (17)	103	ND
Kidney.....	.17	1.00 ± .58 (6)	17	.36	1.50 ± .62 (7)	24	ND
Brain.....	1.17	1.60 ± .56 (9)	73	.47	.52 ± .17 (13)	90	ND

NOTE.—ND = not determined.

[a] mtDNA levels were determined in age-matched control postmortem tissues obtained within 6 h of death from infants who showed no morphological or biochemical abnormality of mitochondria in any of the tissues studied. Controls are expressed as mean ± SD.

[b] COX activity was determined in control tissues from individuals who showed no evidence of morphological or biochemical abnormality of mitochondria in any of the tissues studied. Activities expressed as nmol cytochrome *c* oxidized/min/mg of homogenate protein for patients 1 and 2, and as nmol cytochrome *c* oxidized/min/g of wet tissue for patients 3 and 4. All controls are expressed as mean ± S.D.

[c] Abnormal mitochondrial proliferation was visualized by electron microscopy in patients 1 and 2 (Boustany et al. 1983), or by the modified Gomori procedure (Engel and Cunninghan 1963) in patients 3 and 4.

ysis of COX activity in homogenates showed a profound deficiency only in muscle of patient 1 and in liver of patient 2.

During the course of these studies, two unrelated infant boys (patients 3 and 4) came to our attention. Both patients had severe generalized weakness, lactic acidosis, and respiratory insufficiency, causing death at 7 and 9 mo, respectively. In addition, both had renal dysfunction, and patient 3 had clinical evidence of brain and heart involvement. Muscle mtDNA levels were reduced in both patients to 3% and 17% of controls, respectively (fig. 1, table 1). Nonmuscle tissues were available only from patient 4: the amount of mtDNA was normal in brain, mildly decreased in liver, but severely reduced (17% of normal) in kidney (fig. 1, table 1). Biochemical analysis showed COX deficiency in muscle homogenate of patient 3 and in muscle and kidney homogenates of patient 4 (table 1).

Morphological studies of muscle from patient 3 provided additional evidence of mtDNA depletion. Using total mtDNA as a probe for in situ hybridization, the mtDNA signal was much weaker than in the control (fig. 2*a–c*). Similar results were obtained with monoclonal anti-DNA antibodies, which normally stain muscle nuclei and the cytoplasm in a pattern that correlates with the location of mitochondria (see Patients and Methods). In muscle sections from patient 3, these antibodies stained the nuclei but not the cytoplasm (fig. 2*e*), while in control muscle staining was clear in both nuclei and in mitochondria in the cytoplasm (fig. 2*d*). Identical results were obtained using autopsy muscle from patient 1 (not shown).

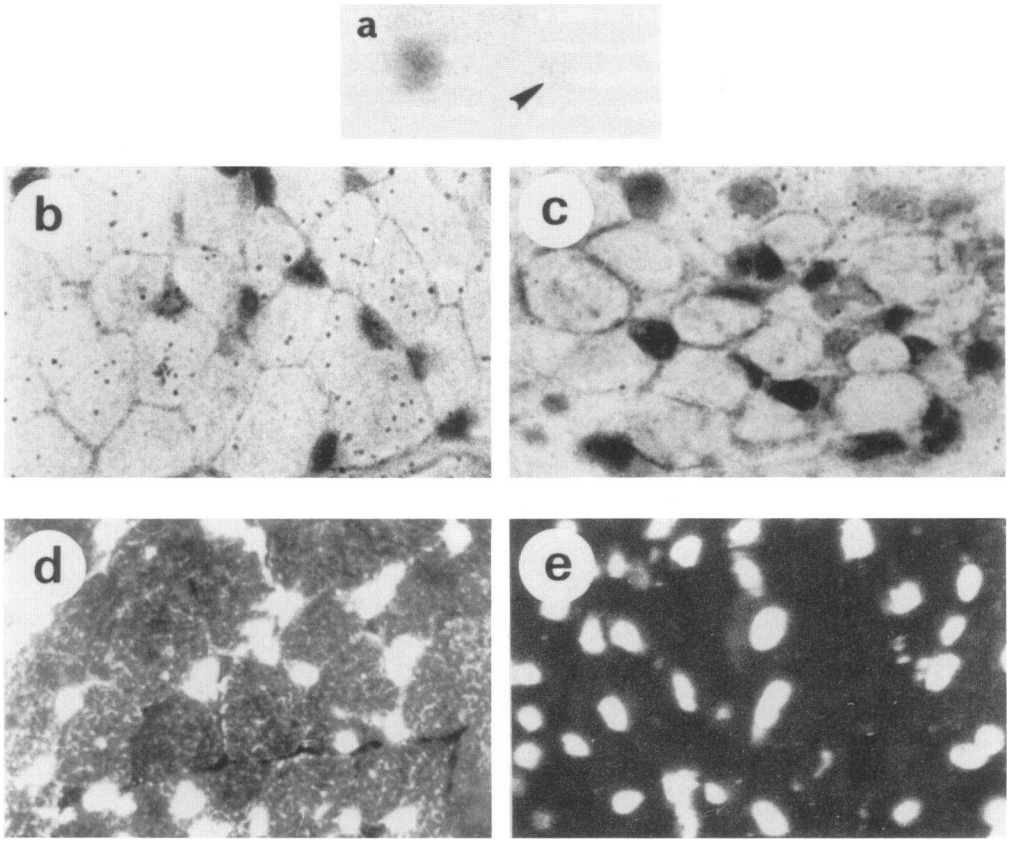

Figure 2 Morphological evidence of muscle mtDNA depletion. Frozen sections of a muscle biopsy from patient 3 were hybridized with ^{32}P-labeled total human mtDNA as described in Patients and Methods. The autoradiogram (*a*) showed a weak signal in the patient's muscle (*right, arrow*) compared to an age-matched control (*left*). This decrease of signal was observed in individual muscle fibers after emulsion development and toluidine blue staining: control muscle (*b*) had a high density of silver grains (although the method was not sensitive enough to differentiate fiber types), while muscle from patient 3 had significantly fewer grains within muscle fibers (*c*). Depletion of mtDNA was also observed by immunostaining muscle sections with an anti-DNA antibody. Cytoplasmic staining, denoting mtDNA, was observed in control muscle (*d*), but not in muscle fibers from patient 3 (*e*). Note the strong nuclear staining in both sections.

To further correlate mtDNA depletion with COX deficiency and with a possible abnormality of mitochondrial protein synthesis, we performed histochemical and immunohistochemical studies on muscle from patient 3. The activity of COX, for which the catalytic subunits are encoded by mtDNA, was markedly decreased as compared to control muscle (fig. 3*a* and *d*), while succinate dehydrogenase, a mitochondrial enzyme encoded exclusively by nuclear DNA, was higher than normal (not shown), reflecting abnormal mitochondrial proliferation. Using a battery of antibodies, we found that two mtDNA-encoded proteins, subunit II of COX (fig. 3*b* and *e*) and subunit I of NADH–coenzyme Q oxidoreductase (not shown), were undetectable in muscle of patient 3, while a nuclear DNA-encoded protein, subunit IV of COX, was present at or above normal levels in fibers with mitochondrial proliferation (fig. 3*c* and *f*).

In an attempt to identify mtDNA mutations that could impair replication, we amplified the L- and H-strand origins of mtDNA replication from muscle of patients 1 and 3 by PCR (Saiki et al. 1988). Direct sequencing of the PCR products showed perfect identity with the published human mtDNA sequence (Anderson et al. 1981), except for what we considered neutral polymorphisms. Both patients had an A-to-G transition at position 263 (numbers according to Anderson et al. 1981), and a length polymorphism in a region corresponding to an evolutionarily conserved sequence box, CSB II (Anderson et al. 1981; Bibb et al. 1981; Anderson et al. 1982), with a C_8TC_6 configuration instead of C_7TC_5 (Anderson et al. 1981). These alterations are

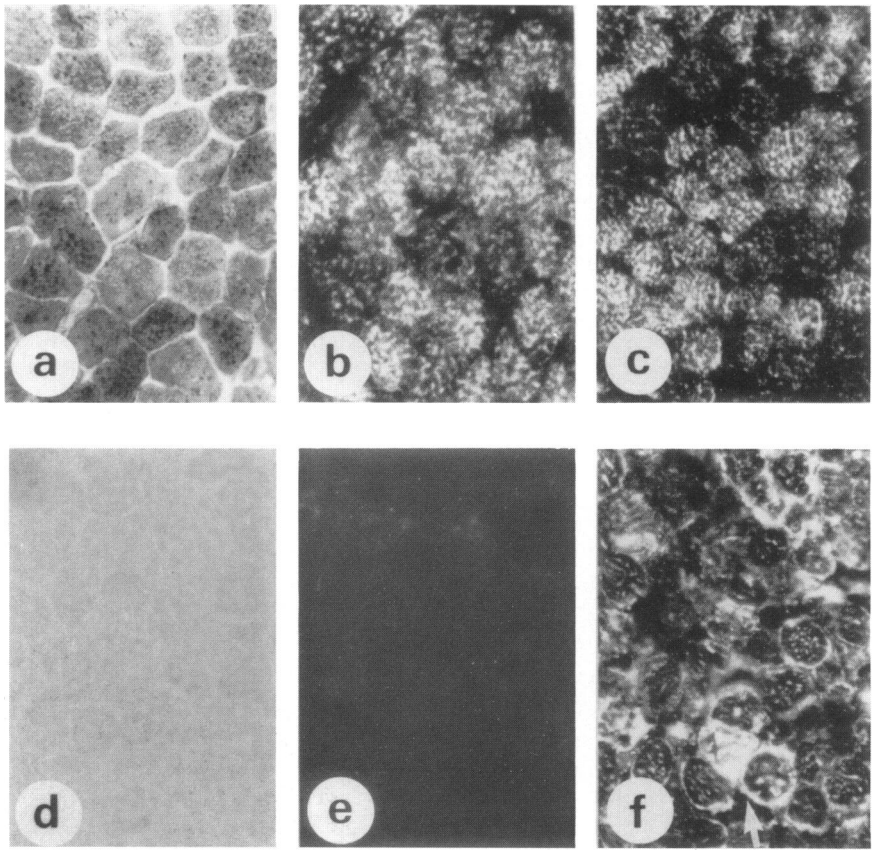

Figure 3 Absence of mitochondrial translation products in muscle of patient with mtDNA depletion. Muscle samples from patient 3 (*lower panels*) and an age-matched control (*upper panels*) were stained for COX activity (*a* and *d*) and immunoreactivity to antibodies against COX subunits II (*b* and *e*) and IV (*c* and *f*). The patient's muscle section showed a marked COX deficiency. The mitochondrially encoded polypeptide COX II was markedly reduced, while the nuclear-encoded polypeptide COX IV showed a strong immunostaining reaction, reflecting a subsarcolemmal accumulation of mitochondria (*arrow*).

considered neutral because they have been observed in normal human tissues (Hauswirth and Clayton 1985; E. Sbisa and C. Saccone, personal communication).

Discussion

We describe here a novel mtDNA abnormality in fatal infantile mitochondrial diseases, in which clinically and biochemically affected tissues had a severe depletion of mtDNA. This was shown by three different methods: quantitative Southern blot analysis, in situ hybridization, and immunohistochemistry. Depletion of mtDNA in these patients seems to be a specific finding and has not been observed previously in other types of mitochondrial diseases. We have performed Southern blot analysis in muscle biopsies from more than 200 patients with diverse mitochondrial disorders (Moraes et al. 1989; S. Shanske and S. DiMauro, unpublished results), and none showed an overt decrease of mtDNA copy number. Included in this series were patients with clinically defined mitochondrial encephalomyopathies such as Kearns-Sayre syndrome (KSS), myoclonic epilepsy with RRFs (MERRF), and Leigh syndrome, as well as patients with pure myopathies, such as the fatal and benign infantile forms of isolated COX deficiency (Moraes et al. 1989). In many of these disorders, which are characterized by RRF, the content of mtDNA in muscle was actually increased, in keeping with the mitochondrial proliferation. Secondary changes, such as muscle degeneration or fibrosis, cannot explain the depletion of mtDNA because these features were not prominent in affected tissues from our patients. Conversely, mtDNA levels were not reduced in muscle biopsies from patients with Duchenne muscular dystrophy, despite extensive muscle degeneration (data not shown).

COX activity was 2% of control in muscle from patient 1, and 7% of control in liver from patient 2 (table 1), and correlated well with the degree of mtDNA depletion in these tissues (2% and 12% of controls, respectively). Similarly, the severe decrease of COX in muscle from patient 3 (1% of control) and in muscle (5%) and kidney (24%) from patient 4 also correlated with the mtDNA depletion in these tissues (3%, 17%, and 17%, respectively). Conversely, tissues which had normal COX activity (e.g., liver and brain in patient 4) had mtDNA levels within the normal range. In tissues where we detected a partial COX deficiency, such as kidney from patients 1 and 2 or liver from patient 1, mtDNA levels were either normal or only partially decreased.

Immunohistochemistry showed the absence of mtDNA-encoded proteins in muscle of patient 3, while COX subunit IV, a nuclear-encoded polypeptide, was present in above-normal levels. These results are consistent with biochemical data in isolated mitochondria from patients 1 and 2, where cytochrome cc_1 (nuclear-encoded) was normal, while cytochromes *b* and aa_3 (mtDNA-encoded) were absent (Boustany et al. 1983; Aprille 1985). The absence of mtDNA-encoded proteins was not surprising given the severely reduced number of mitochondrial genomes. Because mtDNA-encoded proteins are essential for respiratory chain activity, absence of these polypeptides could explain the severe clinical involvement of affected tissues in these patients.

The number of mtDNAs per mitochondrion has been estimated to vary between 2 and 10 in different tissues (Bogenhagen and Clayton 1974; Shmookler Reis and Goldstein 1983; Robin and Wong 1988). If we consider the mtDNA/rDNA ratio observed in our control muscles as a reflection of a maximum of 10 mtDNAs per organelle, muscle from patients 1 and 3 would still have fewer than one mitochondrial genome per mitochondrion (table 1). This value must be even lower, because affected tissues have abnormally increased numbers of mitochondria (Boustany et al. 1983; Aprille 1985; table 1); many organelles must therefore be completely devoid of mtDNA.

A possible explanation for the tissue-specific expression of mtDNA depletion is suggested by diseases associated with heteroplasmic mtDNA deletions, in which differential tissue involvement has been attributed to segregation of normal and mutant mtDNA populations early in development (Holt et al. 1989; Moraes et al. 1989; Nelson et al. 1989; Rotig et al. 1989; Shanske et al. 1990). For example, an identical heteroplasmic mtDNA deletion of 4,977 bp has been found in patients with three different disorders: ocular myopathy, a clinically benign condition (Schon et al. 1989); KSS, a devastating encephalomyopathy (Schon et al. 1989; Shanske et al. 1990); and Pearson syndrome, a disease of bone marrow and pancreas (Rotig et al. 1989). The different clinical presentations seem to be related to the relative proportions of partially deleted mtDNAs, with more mutant mtDNAs in affected than in unaffected tissues. Analogously, the mtDNA depletion with differential tissue expression could be due to a replication-deficient population of mtDNAs that segregated randomly to muscle, liver, or kidney. We sought evidence for such a mutant mtDNA population by sequencing the origins of replication of mtDNA

from affected tissues. Although we did not find a mutation at the origins of mtDNA replication, we cannot exclude this possibility: since the affected tissues are almost completely depleted of mitochondrial genomes, the DNA fragments obtained by PCR could have originated from normal mitochondrial genomes present in nonaffected "contaminating" tissues (fibroblasts or vascular smooth muscle and endothelial cells). In addition, we cannot exclude the possibility of a mutation outside the origins of replication which is nevertheless capable of interfering with replication. Finally, a mutant population representing less than 20% of the total mitochondrial genome would not be easily detectable by direct sequencing of PCR-amplified fragments.

The nonmaternal mode of inheritance in the family of patients 1 and 2 (fig. 1) suggests a mutation in nuclear DNA as the primary cause of the mtDNA depletion. On the other hand, the involvement of different tissues in members of the same family (patients 1 and 2) is difficult to reconcile with a nuclear defect. A nuclear gene abnormality could explain the variable tissue expression if the chromosomal defect elicited a mutation of mtDNA. Just as some autosomal dominant mutations associated with ophthalmoplegia seem to cause multiple deletions in mtDNA (Yuzaki et al. 1989; Zeviani et al. 1989), a mutant nuclear gene might cause an alteration in mtDNA that impairs replication. The mechanisms controlling mtDNA copy number are poorly understood (Clayton 1982; Attardi and Schatz 1988), but several proteins are known to participate in mtDNA replication, including DNA and RNA polymerases, topoisomerases, single-stranded DNA-binding proteins, as well as a mitochondrial RNA-processing ribonucleoprotein (MRP) (Chang and Clayton 1989). A mutation in any of these proteins could also result in mtDNA depletion. It is also conceivable that a "silent" dominant nuclear mutation could be expressed only when combined with a particular mitochondrial genotype. Although no suspicious polymorphism was detected in the regions of mtDNA replication origins, we cannot exclude this possibility.

A third possibility to explain these findings is that the fundamental genetic defect—presumably nuclear-encoded—causes an error in the resumption of mtDNA replication in early embryogenesis. During oogenesis, the number of mitochondria increases about 100-fold, while the number of mitochondrial genomes per organelle decreases from approximately 2–10 to about 1–2 (Piko and Matsumoto 1976; Michaels et al. 1982; Hauswirth and Laipis 1985). After fertilization, the number of mitochondria and mtDNAs in the zygote does not change appreciably until the blastocyst stage (Piko 1970; Cascio and Wassarman 1981; Hauswirth and Laipis 1985; Piko and Taylor 1987), at which time organelle division and mtDNA replication are believed to resume (Piko and Taylor 1987) and somatic cell levels of organelles and genomes are reattained. If, for some reason, organelle division resumed at the appropriate time but the timing of mtDNA replication resumption was delayed beyond the blastocyst stage, it is conceivable that individual stem cell populations could contain abnormally reduced numbers of mtDNAs. If the mtDNA copy number never achieved normal levels during subsequent embryonic and fetal development, a tissue-specific depletion of mtDNA might occur. This hypothesis would also help explain the variable tissue expression of mtDNA depletion among different members of the same pedigree, as the same gene controlling resumption of mtDNA replication might be expressed at slightly different times in different stem cells in individual pedigree members. Because differentiation at the blastocyst/trophoblast stages is an exquisitely timed and highly coordinated process, even subtle differences in the timing and location of the resumption of mtDNA replication could deplete mtDNA in some groups of stem cells while leaving others unaffected.

The mtDNA-depleted cells in affected tissues from our patients resemble ρ^0 mutants, which have been obtained in yeast (Tzagoloff 1982), avian (Desjardins et al. 1986), and human cells (King and Attardi 1989) by prolonged growth in the presence of ethidium bromide. Although vertebrate cells can survive without mtDNA, they become auxotrophic for uridine and pyruvate (Desjardins et al. 1986; King and Attardi 1989). In contrast to cultured cells, absence of mtDNA in human tissues ought to be lethal. While cultured cells can rely largely on glycolysis for energy production, intact organs and tissues depend heavily on the respiratory chain for ATP generation. In agreement with this concept, the mtDNA depletion reported here caused clinical problems soon after birth, with functional failure of affected tissues and early death, presumably because independent aerobic metabolism was severely impaired.

It is still premature to assess the prevalence of this newly described mtDNA depletion phenotype, but biochemical defects affecting multiple complexes of the respiratory chain are not uncommon (Shoffner and Wallace 1990). Some of them have been attributed to large-scale deletions of mtDNA (Holt et al. 1989;

Moraes et al. 1989) or to point mutations affecting tRNA genes (Shoffner et al. 1990). mtDNA depletion provides another genetic basis for mitochondrial diseases associated with combined defects of respiratory chain complexes.

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